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Genotoxic effect of doxorubicin–transferrin conjugate on human leukemia cells

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ABSTRACT

Doxorubicin (DOX) is an effective anthracycline antibiotic against a wide spectrum of tumors and hematological malignancies. It mainly interacts with DNA, but can also generate reactive oxygen species (ROS), which damage cell components. Unfortunately, numerous side effects, such as severe cardiotoxicity and bone-marrow suppression, limit its use. To reduce this obstacle and improve its pharmacokinetics, we conjugated DOX to transferrin (TRF), a human plasma protein. In our study, we compared the effect of DOX and the doxorubicin–transferrin conjugate (DOX-TRF) on human leukemic lymphoblasts (CCRF-CEM), and on normal peripheral blood mononuclear cells (PBMC). In parallel, experiments were carried out on two human chronic myeloid leukemia (CML) cell lines derived from K562 cells, of which one was sensitive and the other resistant to doxorubicin (K562/DOX). By use of the alkaline comet assay, the effect of the agents on the induction of DNA damage in normal human cells and human leukemia cells was determined. Oxidative and alkylating DNA damage were assayed by a slightly modified comet assay that included the use of the DNA-repair enzymes endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (Fpg). To investigate whether DNA breaks are the result of apoptosis, we examined the induction of DNA fragmentation visualized as oligosomal ladders after simple agarose electrophoresis under neutral conditions. Modifications of the genome induced by the different drugs were analyzed following assessment of the cell-cycle phase.

The DOX-TRF conjugate caused more DNA damage than the free drug, the degree of DNA fragmentation being dependent on the duration of treatment and the cell type analyzed. With neutral agarose electrophoresis we showed that the test compounds caused the formation of a characteristic DNA-ladder pattern. Furthermore, the DOX-TRF conjugate generated a higher percentage of apoptotic cells in the subG1 fraction and blocked more cells in the G2/M phase of the cell cycle than did free DOX. In summary, both agents induced DNA damage in cancer cells, but the DOX-TRF conjugate generated more genotoxic effects and apoptosis than the unconjugated drug.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by the BCR-ABL constitutive tyrosine-kinase (TK) oncoprotein, which results from a balanced reciprocal translocation between chromosomes 9 and 22 (*t*(9;22)(q34;q11)) [1,2]. The encoding protein has a constitutive tyrosine-kinase activity and is considered as a pathogenic principle of the Philadelphia chromosome (Ph) identified in nearly 95% of CML [3]. The constitutively activated BCR/ABL tyrosine kinase plays a critical role in the pathogenesis of CML, likely via phosphorylation of multiple downstream protein targets, resulting in the activation of mitogenic cellular pathways and therapeutic resistance. BCR-ABL signaling is responsible for the pathogenesis not only of chronic but also of other types of leukemia [4]. The induction of G2/M cell-cycle arrest, delayed induction of the anti-apoptotic Bcl-xl protein, and activation of DNA repair are thought to be involved in the BCR/ABL-dependent effects. Recent findings indicate that repair of drug-induced DNA lesions may cause drug resistance in BCR/ABL cells [5]. The introduction of the tyrosine-kinase inhibitor STI571 (Gleevec, Imatinib mesylate) in the treatment of chronic myelogenous leukemia has been a major medical breakthrough in the management of this disease [6]. To increase therapeutic efficacy, Gleevec is often applied together with other cytotoxic agents such as the anthracycline drugs doxorubicin (DOX) and daunorubicin (DNR), which have anti-proliferative properties [7]. DOX is a powerful anthracycline antibiotic, used

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to treat many human neoplasms, including acute leukemias, lymphomas, solid tumors and sarcomas [8]. It is a cytostatic drug with multiple modes of action. DOX can inhibit RNA synthesis by binding to RNA polymerase II and topoisomerase II; it may generate reactive oxygen species (ROS) that can cause cell death either by disturbing the cellular redox equilibrium or by damaging the DNA or membranes; and it can intercalate into DNA, preventing replication [9,10]. Doxorubicin may also cause cardiotoxicity when used for a prolonged period of time, which limits its clinical use [11]. The chronic side effects are irreversible and include the development of cardiomyopathy and ultimately congestive heart failure. Various approaches have been developed to circumvent these limitations, including liposome encapsulation, photoactivation and coupling of the drug to various carriers such as monoclonal antitumour antibodies, albumin and transferrin. Carriers that exhibit tumour-cell specificity can in principle be effective at concentrations that do not cause significant systemic toxicity [12,13]. Our previous work demonstrated that coupling doxorubicin to transferrin (TRF) with glutaraldehyde increases the cytotoxicity of DOX and limits multidrug resistance [14]. Taking into account that doxorubicin can generate free radicals and introduce oxidized and methylated bases in DNA, we compared the genotoxicity of free doxorubicin and the doxorubicin transferrin (DOX-TRF) conjugate. We investigated the DNA-damaging potential of the DOX-TRF conjugate in human leukemia cells by use of the comet assay, with leukemic lymphoblasts (CCRF-CEM), chronic myelogenous leukemia cells sensitive (K652) or resistant to doxorubicin (K652/DOX), and normal peripheral blood mononuclear cells (PBMC). We employed the alkaline version (at pH > 13) of the assay in order to reveal DNA single- and double-strand breaks as well as alkali-labile sites. The induction of oxidative damage in DNA by doxorubicin was checked in the presence and absence of the antioxidant *N*-acetylcysteine (NAC). Moreover, in order to assess whether oxidative DNA damage may be induced by the DOX-TRF conjugate, we used two specific enzymes in the comet assay, *viz*. endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (Fpg). The removal of oxidized bases from DNA by Endo III leads to additional DNA single-strand breaks detectable by the comet assay [15]. On the other hand, Fpg is a glycosylase that initiates base-excision repair in *Escherichia coli*. It recognizes and removes 7,8-dihydro-8-oxoguanine (8-oxoguanine), the imidazole ring-opened purines 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-Gua) and 4,6-diamino-5-formamidopyrimidine (Fapy-Ade) as well as 7,8-dihydro-8-oxoadenine [16]. The removal of specific modified bases from DNA by this enzyme leads to formation of apurinic or apyrimidinic sites, which are subsequently cleaved by its AP-lyase activity. The resulting gap in the DNA strand can be detected by the comet assay [17].

So far the genotoxic effects induced by the DOX-TRF conjugate in human leukemia cells have not been investigated by others. In a preliminary study on the genotoxicity of the DOX-TRF conjugate in two human leukemia cell lines, we observed that the mechanism of intracellular transport and the cytotoxic effects of free DOX and its conjugate with TRF were quite different [14]. Therefore, the aim of this study was to obtain further insight into the role of DNA lesions produced in relation to cell death induced by both compounds. In particular, we studied the induction of DNA lesions (including DNA single-/double-strand breaks and oxidative base damage measured by means of the comet assay, and DNA–protein-crosslinks shown in a comet assay modified by a post-incubation of the samples with proteinase K), cell-cycle arrest, and the induction of apoptotic or necrotic cell death. The induction of apoptotic death results in fragmentation of DNA in the form of oligosomal ladders, which was tested by means of agarose-gel electrophoresis performed under neutral conditions. The induction of cell-cycle arrest was determined by means of flow cytometry.

2. Materials and methods

2.1. Chemicals

Doxorubicin DOX was purchased from Sequoia Research Products (Pangbourne, United Kingdom). Fetal bovine serum (FBS), penicillin streptomycin, L-glutamine, phosphate-buffered saline and RPMI-1640 medium, were obtained from Lonza (Lievres, Belgium). Agarose, low melting-point agarose (LMP), normal melting-point agarose (NMP), 4′,6-diamidino-2-phenylindole (DAPI), *N*-acetylcysteine, and all reagents required for the conjugation procedure were obtained from Sigma Chemicals (Darmstadt, Germany). Endo III, and Fpg were kind gifts from Dr. Sabina Tabaczar from the Department of Molecular Biophysics, University of Lodz, Lodz, Poland. The other chemicals were purchased from POCH S.A. (Gliwice, Poland) if not otherwise indicated. Tissue culture dishes were from Corning (New York, USA).

2.2. Cell cultures

Most experiments were carried out on two human chronic myeloid leukemia cell-lines. Both were derived from K562 cells, one was sensitive and the other resistant to DOX. They were obtained from Prof. J. Robert at the Institute Bergonie, France. The human erythroleukemia cell line resistant to DOX (K562/DOX) was grown with 0.02 μM doxorubicin as a selective agent. Experiments were also performed with an in vitro model of acute lymphoblastic leukemia cells (CCRF-CEM), which were a kind gift from Prof. G. Bartosz (Department of Molecular Biophysics, University of Lodz, Poland). The cells were cultured in RPMI medium (Lonza, Belgium) supplemented with 10% fetal bovine serum, and 100 μg/ml penicillin/streptomycin, at 37 °C in a humidified 5% CO_2 atmosphere. Exponentially growing cells, with a viability higher than 95% were used in all analyses. Cells for determination of growth rate were seeded at a density of 1×10^5 per sample and cultured for up to six days. Every 12 h the number of viable cells was evaluated by the trypan-blue exclusion method. In parallel we investigated whether the DOX-TRF conjugate is genotoxic toward normal cells. To this end, peripheral blood mononuclear cells were isolated by density centrifugation (30 min, 300 × *g*, 22 °C) on Histopaque (Sigma, Germany). Blood samples were obtained from young (23–25 years), non-smoking healthy men. The viability of the cells was about 90%. The final concentration of the cells was adjusted to 10^5 cells per ml by adding the appropriate volume of RPMI 1640 without glutamine to the single-cell suspension.

To confirm the role of reactive oxygen species in the induction of DNA damage by the DOX-TRF conjugate, experiments were performed in the presence or absence of 3 mM NAC. The antioxidant was added directly to the cell medium 1 hour before drug treatment. In all experiments, cells were treated with the IC_{50} drug concentration. These dosages of analyzed compounds, estimated by the XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay, were presented in our previous work: DOX: CCRF-CEM – 0.13 μM, K562 – 0.27 μM, K562/DOX – 2.60 μM, PBMC – 0.57 μM; DOX-TRF: CCRF-CEM – 0.057 μM, K562 – 0.072 μM, K562/DOX – 0.26 μM, PBMC – 1.13 μM) [14].

2.3. Comet assay

To examine DNA damage, the comet assay was performed under alkaline conditions essentially according to the procedure of Singh [18] with some modifications [19]. The cells were treated with DOX-TRF conjugate and free DOX at the IC_{50} concentration for up to 72 h (3, 6, 12, 24, 48, 72 h). In some samples, the cells were preincubated with NAC to analyze the contribution of free radicals to DNA damage induction. Peripheral blood mononuclear cells treated with 10 μM hydrogen peroxide for 10 min at 4 °C served as positive control. After electrophoresis and DAPI staining, the slides were analyzed at 200× magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, Inc., San Diego, CA) equipped with a UV filter-block and connected to the image-analysis system Lucia-Comet v. 4.51 (Laboratory Imaging, Praha, Czech Republic). Fifty images from cells were selected from each sample and each experiment was repeated three times. The percentage DNA in the tail was determined [20], and the mean value of the % tail DNA was taken as an index of DNA damage in a sample. In the samples preincubated with NAC, the level of DNA damage associated with free-radical formation was determined.

2.4. Detection of DNA–protein cross-links

For analysis of the formation of DNA–protein cross-links – which are characteristic for programmed cell death – the cells treated with DOX-TRF conjugate or free DOX were processed in the comet assay as described above, except that proteinase K treatment was carried out after the lysis [21]. Slides were washed three times with TE buffer (10 mMTris, 1mMEDTA, pH 10), covered with 100 μl proteinase K (1 mg/ml TE buffer) and incubated for 2 h in a moist chamber at 37 °C. Controls were incubated with 100 μl TE buffer only. After removal of the cover slip, the slides were processed as described in Section 2.3.